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Therapeutic effect of D-Carvone on inflammation, apoptosis, and cell damage in lithium-induced liver injury model in rats

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ABSTRACT

Objectives: Lithium is an element denoted by the symbol Li in the periodic table. Lithium salts are widely used worldwide as therapeutic agents in treating excitement. However, the use of lithium causes liver damage. Carvone is an unsaturated monoterpenoid ketone usually found in essential oil extracts of aromatic medicinal plants such as dill, mint, and cumin. Various studies have shown that D-Carvone has strong antioxidant and anti-inflammatory effects. This study aimed to investigate the therapeutic effect of D-carvone on apoptosis, inflammation, and cell damage in lithium-induced liver injury.

Materials and Methods: The rats in our study were divided into 4 groups control, D-Carvone, Lithium, and Lithium+D-Carvone. After the treatments, rats were decapitated and liver tissues were removed. Histopathological analyses were performed on liver tissue and Bcl-2, Bax, P2X7R, and NfκB-p65 expression levels were evaluated by the Western Blot method.

Results: We determined that lithium administration caused liver tissue damage and increased Bax, P2X7R, and Nfkβ-p65 expression and decreased Bcl-2 expression. D-Carvone administration ameliorated these changes.

Conclusions: As a result, it was observed that D-Carvone administration ameliorated lithium-induced liver tissue damage and showed this effect by suppressing the inflammatory and apoptotic process.

Keywords: *Lithium, D-Carvone, Bcl-2, Bax, P2X7R, Nfkβ-p65*

INTRODUCTION

Lithium is a soft metallic element, denoted by the symbol Li in the periodic table. Lithium-ion batteries and lithium carbonate tablets for mood stabilization are the two best-known applications of lithium. Clinically, lithium has been used as a classical mood stabilizer in the treatment of bipolar disorder for more than fifty years (Johnson and Gershon, 1999).

The very strong anti-suicide effect of lithium has also been recently demonstrated (Tondo and Baldessarini, 2009). Lithium salts are widely preferred worldwide as therapeutic agents for treating excitement and mania. Lithium salts are easily distributed in the body, easily absorbed from the intestine and almost all of them are excreted through the urinary system (Rosenthal and Goodwin, 1982). Long-term lithium use has been found to have adverse effects on various organs, especially the liver. Numerous reports reflecting the toxic effects of lithium on liver function and structure have been put forward (Hunt et al., 1983; Abbas and Kathem, 2021; Bouyahya et al., 2021). It

has been suggested that lithium is a xenobiotic and affects the hepatic drug-metabolizing enzyme system (Hunt et al., 1983).

Carvone is an unsaturated monoterpenoid ketone usually found as the main phytochemical component in essential oil extracts of aromatic medicinal plants such as dill, mint, and cumin, with a boiling point of 230°C (Abbas et al., 2020; Zhao and Du, 2020; Bouyahya et al., 2021). Carvone has two enantiomers that differ in their biological properties, Carvone (L-Carvone) found in mint leaves and Carvone (D-Carvone) found in cumin leaves (Alsanea and Liu, 2017).

Pharmacologically, D-Carvone has been found to have anti-hyperlipidemic, fungicidal, anti-cancer, immunomodulatory, antioxidant, antimicrobial, anti-hypertensive, and anti-inflammatory effects (Lv et al., 2021). It was reported that D-Carvone showed no toxic effects when taken at 0.6 mg/kg body weight/day (Mahboubi, 2019). In mice fed a fatty diet, D-Carvone was found to reduce lipid accumulation by regulating gene expression of proteins responsible for lipid synthesis and transport in the liver. In another study, it was found that Carvone decreased hepatic cholesterol and triglyceride levels and decreased hepatic steatosis in rats with non-alcoholic fatty liver disease (Günther et al. 2019). The present study aimed to investigate the ameliorative effects of D-Carvone against lithium-induced hepatocyte damage.

MATERIALS and METHODS

Animal and experimental procedure

All studies were approved by Bingöl University Animal Experiments Local Ethics Committee (Decision no: BÜHADYEK-01/09). Adult 12-week-old male rats (n=32) obtained from BÜDAM were randomly divided into 4 groups of 8 rats per group based on body weight and housed in separate cages at an ambient temperature of 22 (±2)°C. The light regime was set to 12 hours light and 12 hours dark cycle. Rats in all groups were allowed free access to food and water. The lithium chloride (LiCl) supplemented feeds used in the experimental study were prepared by a commercial company that prepares certified experimental animal feed at a rate of 40 mmol LiCl per 1 kg dry feed (40 mmol/kg feed). From the beginning to the end of the experimental study, housing, feeding and care of all rats and all experimental procedures were carried out at BÜDAM.

Table 1. Groups and applications.

Group Name	Day 0-28. (n=8)	Day 28-42. (n=8)
Group 1: Control (CNT, n=8)	For 28 days, rats were fed with unadulterated standard commercial feed.	Rats were fed with standard commercial feed without additives.
Group 2: D-Carvone (D-CAR, n=8);	For 28 days, rats were fed with unadulterated standard commercial feed.	Rats were fed with unadulterated standard commercial feed and at the same time, each rat in the group was given 20 mg/kg D-carvone intraperitoneally daily for 14 days.
Group 3: Lithium (LIT, n=8);	Rats were fed 1 kg of standard commercial feed supplemented with 40 mmol Lithium chloride (LiCl) for 28 days.	Rats were fed with 1 kg standard commercial feed supplemented with 40 mmol Lithium chloride (LiCl).
Group: Lithium+D-Carvone (LIT+D-CAR, n=8)	Rats were fed 1 kg of standard commercial feed supplemented with 40 mmol Lithium chloride (LiCl) for 28 days.	Rats were fed with 1 kg of standard commercial feed supplemented with 40 mmol LiCl and 20 mg/kg D-carvone were administered intraperitoneally daily to each rat in the group for 14 days.

From the beginning of the study, except for the rats in the Control and D-Carvone groups, the other groups were fed with rat pellet feed supplemented with 40 mmol LiCl for 42 days. Rats in the D-Carvone group were fed with standard rat chow for 42 days and 20 mg/kg D-Carvone was administered intraperitoneally for 14 days after the 28th day. Rats in the LiCl+D-Carvone group were

fed with rat pellet feed supplemented with LiCl for 28 days and then 20 mg/kg D-carvone was administered intraperitoneally every day for 14 days. The groups formed within the scope of the study and the experimental procedure applied to the rats in each group are presented in Table 1.

Completion of the study

At the end of the 42-day experiment, the rats in all groups were weighed and their body weights were determined. Following general anesthesia, blood samples were collected from the intracardiac, and decapitation was performed. Blood samples were collected into serum tubes with yellow caps and centrifuged at 4000 rpm for 5 minutes and the sera obtained were stored in a deep freezer at -80°C.

After blood samples were taken, liver tissues were taken and their weights were determined. Some of the liver tissue samples were immersion fixed in 10% buffered formaldehyde solution for histopathological analysis and some were stored in a -20°C deep freezer for the first 24 hours and then in a -80°C deep freezer for Western-Blot analysis.

Histopathologic analysis and evaluation

Liver tissue samples were fixed in 10% buffered neutral formalin and after routine histological technique procedure embedded in paraffin. Paraffin blocks were sectioned at 5 µm thickness and stained with Mallory's triple stain modified by Crossman. They were then evaluated using a light microscope (Zeiss AXIO Scope.A1, German) with 200x magnification. Histologic photomicrographs were converted to quantitative analysis using a scoring system (Niazvand et al., 2023). Depending on the extent of sinusoidal dilatation, degeneration, congestion, and hemorrhage, the results were scored as 0, 1, 2, and 3, representing normal, mild, moderate, and severe damage, respectively.

Western blot analysis

Before western blot analysis, the acquired hepatic tissue samples were kept at -80 °C in a deep freezer. The hepatic tissue samples were weighted and crushed in nitrogen gas, treated with radioimmunoprecipitation (RIPA buffer, Ecotech Bio, Turkey) supplemented with protease and phosphatase inhibitors, and homogenized using a tissue lyser device (Qiagen, USA) at 30 Hz for 20 sec to determine the relative protein expressions of Bcl-2 (sc-7382, Santa Cruz), Bax (Sc-7480, Santa Cruz), P2X7R (11144-1-AP, Proteintech), and NfκB-p65 (sc-109, Santa Cruz). A protein assay kit was used to quantify hepatic tissue's total protein (Pierce BCA, Thermo Sci., USA). 30 ug of protein were then put into the PVDF membrane after being separated by 10% SDS-PAGE. First, at room temperature, 5% bovine serum albumin was used to block the membranes for 90 minutes. Then, the membranes were incubated at 4°C overnight with the appropriate primary antibodies. After primary antibody incubation, the PVDF membranes were washed with TBST and then incubated for an additional 90 minutes at room temperature with the second antibody (Santa Cruz, sc-2004/sc-2005) coupled to horseradish peroxidase. Then, the protein bands were captured using the enhanced chemiluminescence reagent Western ECL substrate (Thermo, 3405), visualized, and analyzed by Image Lab™ Software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Statistical analysis was performed using the SPSS (version 25.0; IBM SPSS Inc, Chicago, IL, USA) package program. Descriptive statistical analyses (mean ± standard deviation) were used. One-way ANOVA test and post hoc Tukey test were performed to compare groups. P values less than 0.05 at the 95% confidence interval were considered statistically significant.

RESULTS

Histopathologic evaluation

Photomicrographs of liver tissue in different groups are presented in Figure 1.

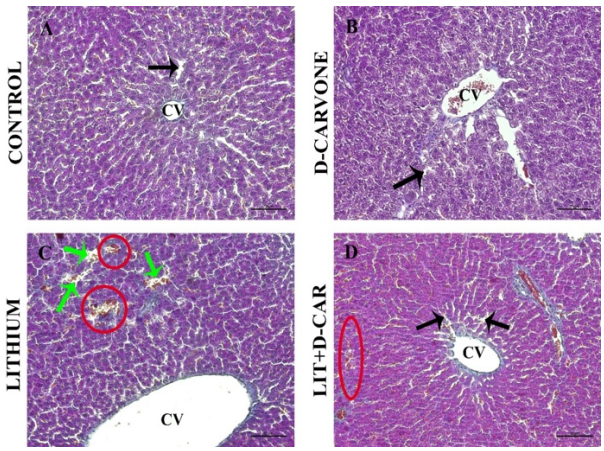

Figure 1. Photomicrographs of liver tissue in different groups. A: Control, B: D-Carvone, C: Lithium, D: Lithium+D-Carvone. Central vein (CV), sinusoid (black arrow), sinusoidal congestion and bleeding (red circle), and sinusoidal dilatation (green arrow). Staining: Mallory's triple stain modified by Crossman, magnification: 200X.

The control group (Figure 1-A) showed normal histologic architecture of the lobules (sinusoids, hepatocytes, and Kupffer cells) in the liver. No

changes were observed in the liver architecture in the D-Carvone (D-CAR) group (Figure 1-B). Figure 1-C shows the liver tissue in the Lithium (LIT) group. Lithium administration resulted in irregularities in the sinusoids such as dilatation, degeneration, congestion, and hemorrhage. On the other hand, the group treated with Lithium and D-Carvone showed reduced liver damage compared to the group treated with only Lithium (Figure 1-D). The damage in these groups was characterized by mild dilatation and congestion of some blood sinusoids. The severity of damage in all groups is listed in Figure 2 according to the defined scoring.

Western blot analysis results

When the relative protein expression levels of liver tissue were evaluated, Bax, NfκB-p65, and P2X7R expression levels were low in the control and D-Carvone groups and there was no significant difference between them ($p>0.05$). However, Bax, NfκB-p65, and P2X7R expression levels were significantly increased in the lithium-treated group compared to the other groups ($p<0.05$). In the lithium+D-Carvone group, these protein expressions were significantly decreased compared to the lithium group ($p<0.05$). On the other hand, the Bcl-2 expression level was higher in the control and D-Carvone groups compared to the other groups and there was no significant difference between them ($p>0.05$). We determined that the Bcl-2 expression level was significantly decreased in the lithium-treated group compared to the other groups ($p<0.05$). In the lithium+D-Carvone group, the expression of this protein was significantly increased compared to the lithium group ($p<0.05$), (Figure 3).

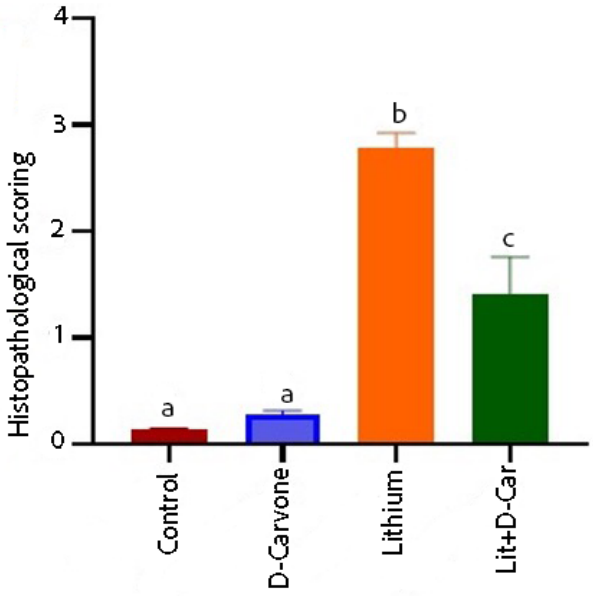

Figure 2. Assessment of liver histology. The values are given as mean ± SD (n=6) and analyzed by one-way ANOVA followed by the Tukey test. The letters (a, b, c) indicate statistically significant difference between the groups, $p<0.05$

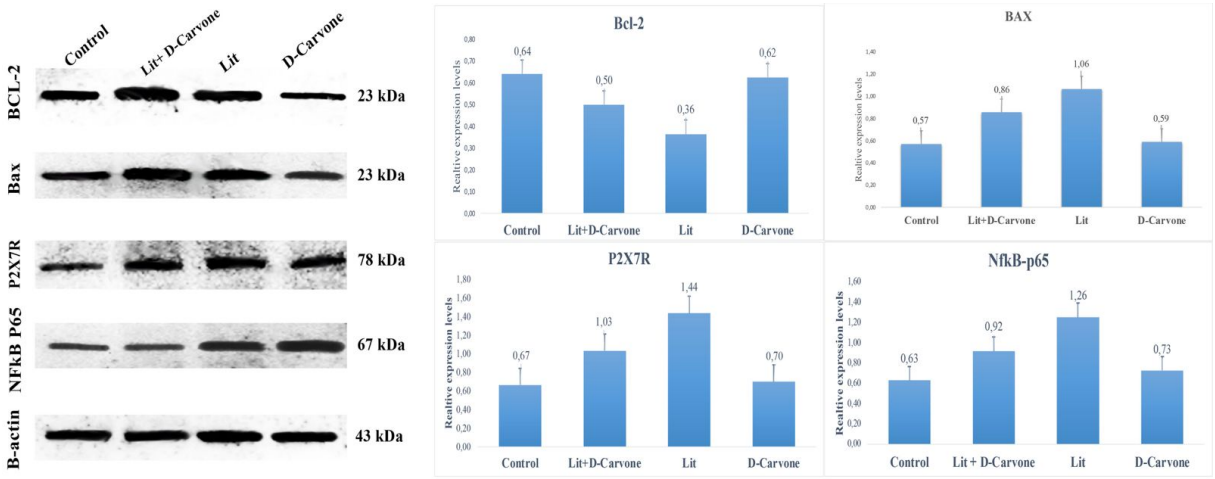

Figure 3. Relative expression of proteins for Bcl-2, Bax, P2X7R, and NfκB-p65. The values are given as mean ± SEM (n=7) and analyzed by one-way ANOVA followed by the Tukey test, $P<0.05$.

DISCUSSION

Lithium salts are a widely used agent in excitement control worldwide. When taken by mouth, lithium is easily absorbed from the intestines, easily distributed throughout the body, and excreted through the kidneys (Pert et al., 1978; Rosenthal and Goodwin, 1982). Long-term treatment with

lithium has been found to cause adverse effects on various organs including the liver (Laakso and Oja, 1979; Casado et al., 1989). There are various reports reflecting the toxic effects of lithium on liver structure and function (Ghoshdastidar, 1990; Lydiard and Gelenberg, 1982). Lithium, a xenobiotic, has been shown to affect the hepatic drug-metabolizing enzyme system (Schou et al., 1968; Aniya and Matsusaki, 1983; Hunt et al., 1983). As a result of various studies, it has been reported that lithium causes cellular damage in liver tissue and stimulates apoptosis and inflation (Tandon et al., 1997). This study aimed to determine the therapeutic effect of D-carvone, which is reported to have antiapoptotic and anti-inflammatory effects (Zhu et al., 2020; Engin et al., 2023), on cellular damage, apoptosis, and inflammation caused by lithium.

In the study, dilatation, degeneration, congestion, and hemorrhage in the sinusoids of hepatocytes in lithium-treated animals were significantly damaged compared to normal rats. These histopathologic changes may be explained by the fact that lithium exerts its toxic effects primarily through the formation of reactive oxygen species, resulting in damage. The necrotic conditions observed in the liver of lithium-treated animals are in agreement with the molecular changes observed. Previous studies are consistent with our findings (Ben Saad et al., 2017; Dai et al., 2020). Co-administration of D-Carvone with lithium-treated animals resulted in a remarkable normalization of hepatic changes. Such hepatoprotective effects of D-Carvone have been observed in several previous studies (Mezni et al., 2022). The data obtained in the present study are similar to previous studies.

The transcription factor NF-κB regulates many aspects of innate and adaptive immune functions and serves as an important mediator of inflammatory responses. Nf-κB induces the expression of several pro-inflammatory genes, including those encoding cytokines and chemokines, and also participates in inflammatory regulation (Erbaş et al., 2024). In addition, NfκB plays a critical role in regulating the survival, activation, and differentiation of innate immune cells and inflammatory T cells (Mohamed and Younis, 2022). In a study, it was reported that there was a significant increase in NfκB levels due to lithium administration. As seen in the present study, NfκB levels analyzed in liver tissues showed a significant increase in the lithium-treated group compared to the control group (Liu et al., 2017). In another study, it was shown that D-Carvone administration maintained NfκB levels at physiologic levels (Makola et al., 2021; Sousa et al., 2023). In the present study, NfκB levels, which increased due to lithium administration, remained at normal physiologic values in rats administered lithium and D-Carvone as in the control groups.

Anti-apoptotic Bcl-2 and pro-apoptotic Bax give us information about whether apoptosis occurs in cells (Ogaly et al., 2022; Gelen et al., 2024). In the study, it was observed that the Bax level was significantly higher than the control due to lithium administration. It was observed that Bcl-2 level, which has anti-apoptotic properties, decreased due to lithium administration. In a study, it was observed that tissue damage occurred in rats given lithium and accordingly, the anti-apoptotic Bcl-2 level decreased and the proapoptotic Bax level increased (Neamatallah et al., 2018). Previous studies reported that D-Carvone administration was effective on Bax and Bcl-2 (Alural et al., 2015). In the present study, it was determined that Bax and Bcl-2 levels in rats administered D-Carvone together with lithium remained close to the control and there was no statistical difference between them and the control.

The P2X7 receptor mediates oxidative stress, inflammatory mechanisms (Gopalakrishnan et al., 2019; Kara and Özkanlar, 2023), and fibrogenesis (Das et al., 2013) in the liver. This receptor triggers Kupffer cell inflammatory cellular responses and hepatocyte damage in liver diseases. In some studies, P2X7 receptor expression was found to be increased in the liver of septic mice. Furthermore, AST and ALT enzymes released by hepatic cell disruption are decreased in the serum of septic P2X7-deficient mice, providing evidence for the importance of this receptor in sepsis-related liver injury. Accordingly, P2X7-deficient mice showed a significant reduction in serum ALT levels in experimental models of acetaminophen-induced acute liver injury (Huang et al., 2014) and nonalcoholic steatohepatitis (Gopalakrishnan et al., 2019). It is also in agreement with a previous study showing impaired glucose metabolism in P2X7-deficient mice resulting in hepatic injury with higher glycemia, dyslipidemia, increased susceptibility to glucose intolerance, and insulin resistance (Hoque et al., 2012; Arguin et al., 2017). In our study, we found that lithium administration increased P2X7 receptor expression in liver tissue, which is an indication that lithium causes damage in liver tissue. We also showed that D-Carvone

administration suppressed lithium-induced P2X7 receptor expression.

CONCLUSION

In conclusion, our data show that lithium administration induces cellular damage, apoptosis, and inflammation in liver tissue. On the other hand, D-carvone administration inhibits lithium-induced tissue damage, inflammation, and apoptosis. This effect of D-carvone was thought to be due to its antioxidant and anti-inflammatory activity.

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